APPLIED MICROBIOLOGY, Jan. 1972, p. 54-61
Vol. 23, No. 1
Copyright © 1972 American Society for Microbiology
Printed in U.S.A.

Immunofluorescence Staining of Group B Coxsackieviruses

MORRIS L. V. FRENCH, NATHALIE J. SCHMIDT, RICHARD W. EMMONS, AND EDWIN H. LENNETTE

Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley, California 94704

Received for publication 20 August 1971

Studies were conducted on the sensitivity and specificity of indirect fluorescent-antibody (FA) staining for identification of group B coxsackieviruses. Antisera produced in four different species (monkeys, rabbits, horses, hamsters) and immune ascitic fluids prepared in mice were compared for suitability in FA staining. The horse antisera showed high titers of nonspecific staining, and the rabbit antisera showed relatively low homologous FA titers. Immune reagents from monkeys, hamsters, and mice were used for homologous and heterologous testing against cell cultures infected with the various group B coxsackieviruses. Antisera or immune ascitic fluids produced in these three species showed some heterotypic and nonspecific staining at low dilutions, with the monkey antisera showing the highest heterotypic titers. However, the immune reagents could be diluted to a point where they gave no heterotypic reactivity, but still showed characteristic homotypic staining. Heterotypic staining appeared as diffuse, low-level staining of the cells, whereas homotypic staining revealed characteristic, brightly staining aggregates of viral antigen in the cytoplasm of the infected cells. By using hamster immune sera, appropriately diluted to eliminate heterotypic staining and yet give strong homotypic staining, it was possible to identify correctly 79 (93%) of 85 field strains of group B coxsackieviruses at the first passage level in BS-C-1 cells; the remainder of the strains were identified after two passages in BS-C-1 cells. No incorrect identifications were made. A limited number of field strains of group B coxsackieviruses were passed into rhesus monkey kidney and human fetal diploid kidney cells, and these were all correctly identified by FA staining, even the strains which failed to produce a cytopathic effect in the human fetal diploid kidney cells. Two human heart and brain tissues from which coxsackievirus type B4 had been isolated failed to show homotypic FA staining in excess of nonspecific or heterotypic staining.

Immunofluorescence staining of the coxsackieviruses has received increased attention in the past few years both for the identification of viruses isolated in cell cultures (6, 11, 17) and for the direct demonstration of viral antigen in human cells and tissues (1-4, 18, 20).

Shaw et al. (17) reported on the identification of certain coxsackieviruses and echoviruses by direct fluorescent-antibody (FA) staining using rabbit antisera combined into pools according to the scheme of Lim Benyesh-Melnick (8) and conjugated with fluorescein isothiocyanate. The conjugates were absorbed with rabbit brain and liver powders and monkey kidney (MK) cells, and this, together with the use of rhodamine B for a counterstain, reduced nonspecific staining to the extent that type-specific identification of the enteroviruses could be achieved. Hatch (6) also utilized the direct FA technique for the type-specific identification of coxsackieviruses isolated in MK cells, but with individual, rather than pooled, coxsackievirus antisera. The conjugated antisera were absorbed with monkey liver powder and MK cells to reduce nonspecific staining.

Sommerville has employed the indirect FA technique with monovalent and polyvalent group B coxsackievirus antisera prepared in rabbits to demonstrate coxsackievirus antigen in human leukocytes (20) and cellular deposits from cerebrospinal fluids (18). He reported

that nonspecific fluorescence could be minimized by using conjugated anti-rabbit globulin prepared in mice (19).

Type-specific staining of certain group A coxsackieviruses propagated in human amnion cell cultures was demonstrated by Zalan et al. (21) using the indirect FA procedure with rabbit coxsackievirus antisera. Nonspecific staining was reduced by absorption of the antisera with mouse liver powder and human amnion cells.

In contrast to the type-specific FA staining reactions reported by the above investigators, Chaudhary and Westwood (5) have described extensive cross-reactions within and between the poliovirus, coxsackievirus, and echovirus groups. They propagated viruses in grivet MK cells and used the indirect FA staining technique with rabbit antisera and an anti-rabbit conjugate prepared from guinea pig serum.

Burch and co-workers, using the direct FA technique, have demonstrated staining of kidney tissue from patients with renal disease (2, 3) and of heart tissue taken at autopsy (4) with conjugated rabbit antisera to certain of the group B coxsackieviruses. In most instances, staining was demonstrated with antisera to more than one group B coxsackievirus type.

The specificity of FA staining with the group B coxsackieviruses should be more clearly defined; this would aid in assessing the significance of staining reactions which may be observed with human tissues. The group B coxsackieviruses have been shown to share common antigens (13, 16) which can be detected in immunodiffusion or complement fixation tests using human sera or sera from monkeys infected with the viruses by the oral route. The role which these group antigens may play in FA staining reactions should be clarified.

The present investigations were concerned with determining the sensitivity and specificity for FA staining of group B coxsackievirus antisera produced in various species and with further evaluation of the FA-staining technique for identification of coxsackieviruses isolated in cell culture systems.

MATERIALS AND METHODS

Virus strains. Prototype virus strains (10) of group B coxsackievirus types 1 to 6 and echovirus type 9 were employed for preparation of immune sera and as controls for FA staining. Field strains of coxsackieviruses and other enteroviruses were isolated and identified by the standard procedures of this laboratory (12).

Cell cultures. The BS-C-1 line of grivet MK cells, secondary rhesus monkey kidney cells, and human fetal diploid kidney (HFDK) cells were propagated by methods described elsewhere (12).

Coxsackievirus immune sera. Hamsters and rabbits were inoculated with equal parts of adjuvant (1 part of Arlacel A and 9 parts of standard mineral oil, C.T.70) and a 10% suspension of infected mouse brain or muscle in phosphate-buffered saline (PBS) or with serum-free, infected MK cell culture fluid in the case of rabbits immunized with virus types B4 and B5. The hamsters received 1 ml and the rabbits received 5 ml of the inoculum by the intraperitoneal route at weekly intervals for a total of three inoculations. The animals were bled 10 to 14 days after the last immunizing injection. Monkey immune sera were prepared as previously described (14) by intramuscular inoculation of rhesus monkeys with a mixture of equal volumes of infected MK cell culture fluid and adjuvant. The enterovirus immune horse sera (9) were obtained from the World Health Organization International Reference Center for Enteroviruses, Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Tex.

Immune ascitic fluids for group B coxsackieviruses types 1 to 6 were produced in adult, female Swiss-Webster white mice (Rockefeller Foundation strain). The immunizing antigen consisted of a 20% suspension (in PBS) of infected suckling mouse brain (for types B1-B5) or muscle (for type B6) emulsified with an equal volume of adjuvant [1 part of Arlacel A, 9 parts of standard mineral oil, C.T. 70, and 0.5 mg of *M. butyricum* (Difco)/100 ml]. Before immunization, the animals were bled from the retro-orbital plexus and then inoculated by the intraperitoneal route with 0.3 to 0.5 ml of the virus-adjuvant mixture on days 0, 7, and 14. On day 17, the animals were inoculated intraperitoneally with 0.2 ml of a 10% suspension (in PBS, *p*H 7.3) of freshly-harvested 180/TC mouse sarcoma cells. The ascitic fluids were aspirated on day 25 and every 3 to 4 days thereafter for a total of five to six times. Each immune ascitic fluid had a neutralizing antibody titer of 1:128 or greater against the homologous virus, and showed no neutralizing activity for heterologous group B coxsackieviruses. Normal mouse ascitic fluid was produced in the same manner using uninfected mouse tissue suspensions for immunization.

Indirect immunofluorescence staining technique. Cell cultures in 2-oz prescription bottles were inoculated with 1 ml of virus suspension and incubated at 35 C until the monolayers showed a 2 to 3+ viral cytopathic effect (CPE). Cells from infected cultures were dispersed with a 0.25% trypsin 0.05% ethylenediaminetetraacetic acid (EDTA) solution, and the cells from each culture were resuspended in 0.05 to 0.1 ml of medium consisting of 2% fetal bovine serum in Eagle's minimum essential medium (MEM). Smears were prepared by placing small drops of the cell suspension onto microscope slides. After drying at room temperature, the smears were fixed in acetone for 10 min and dried at room temperature. The slides were then stored at −70 C. Prior to FA staining, the slides were allowed to dry at room temperature, and the smears were outlined with quick-drying paint (Tri-Chem pen, Tri-Chem, Inc., Belleville, N.J.).

Twofold dilutions (starting at 1:8) of immune sera or normal control sera were prepared in a 20% suspension of normal beef brain (in PBS), and a drop of each serum dilution was applied to a smear of infected cells and to a smear of uninfected cells for control purposes. The tests were incubated at 36 C in a humidified atmosphere for 20 min. The slides were washed twice (10 min each washing) in PBS, *p*H 7.2 to 7.5, rinsed in distilled water, and allowed to dry at room temperature. A "working dilution" of the appropriate fluorescein-conjugated anti-species globulin was then added to each smear; the conjugate was also diluted in a 20% normal beef brain suspension. Antisera against horse, monkey, and mouse globulins were prepared in rabbits, and antisera against hamster and rabbit globulins were prepared in goats. The immune globulins were conjugated with fluorescein isothiocyanate by a method described elsewhere (7). After incubation at 36 C for 20 min, the slides were washed twice (5 min each washing) in PBS, rinsed in distilled water, and then drained and mounted in a phosphate-buffered glycerol-saline solution (one part of glycerol in three parts of PBS, *p*H 7.2).

Examinations for immunofluorescence staining were made with a Zeiss fluorescent binocular microscope illuminated with an Osram HBO 200 mercury burner; ×8 oculars and a ×40 (dry) objective were used. The filter system consisted of a GG4 Zeiss 41 barrier filter and a UG2 exciter filter. Nonfluorescent immersion oil (type A) was used between the condenser and slide.

The degree of fluorescence was expressed as 1, 2, 3, or 4+. A reading of 4+ indicated glaring yellow-green fluorescence, and 3+ indicated bright green but not glaring fluorescence. 3 or 4+ staining reactions characteristically revealed aggregates of viral antigen in the cytoplasm of the infected cells. A reading of 2 or 1+ indicated dull green fluorescence, with the cells showing diffuse staining.

RESULTS

Homologous immunofluorescence staining with coxsackievirus immune sera produced in various species. Group B coxsackievirus immune sera produced in monkeys, rabbits, horses, and hamsters and immune ascitic fluids produced in mice were compared for ability to stain BS-C-1 cells infected with the homologous virus. As controls, normal sera from each species were tested for possible staining, and uninfected BS-C-1 cells were stained with immune sera and normal sera. The results shown in Table 1 with immune sera to coxsackievirus type B1 are representative of those obtained with all of the group B coxsackieviruses.

At a 1:8 dilution, both normal sera and immune sera tended to show 1 or 2+ staining against infected and uninfected cells. Except in the case of the horse antisera, staining with normal sera and uninfected cells was generally not seen at serum dilutions of 1:16 and higher. The rabbit antisera showed very low homologous titers, barely exceeding the staining of uninfected cells. The horse antisera had higher homologous titers, but stained uninfected cells almost to the same degree as cells infected with homologous virus. Antisera produced in monkeys and hamsters and immune ascitic fluids produced in mice showed specific staining at high dilutions, and little staining with uninfected cells, and thus they were used for further studies on cross-reactivity of the sera and for the identification of coxsackievirus isolates.

A staining reaction of 3 or 4+ was selected as an end point in further studies. This type of reaction showed brilliant fluorescence staining of aggregates of viral antigen in the cytoplasm of infected cells, which was characteristic of specific staining with all of the group B coxsackieviruses (see Fig. 1), and was readily distinguishable from the diffuse, dull green staining of cells sometimes seen with low dilu-

TABLE 1. *Homologous indirect fluorescent-antibody staining with coxsackievirus type B1 immune sera produced in various species*

Species of serum	Degree of fluorescence with serum dilutions[a]					
	Normal BS-C-1 cells		Cox[b] B1 infected BS-C-1 cells			
	1:8	1:16	1:8	1:16	1:32	1:64
Monkey						
Normal	1+	±	2+	±		
Cox B1 immune	2+	0	4+	4+	4+	4+
Rabbit						
Normal	±	0	±	0		
Cox B1 immune	2+	0	3+	3+	2+	0
Horse						
Normal	2+	1+	±	0		
Cox B1 immune	2+	2+	2+	2+	1+	1+
Hamster						
Normal	0	0	0	0		
Cox B1 immune	2+	1+	4+	4+	3+	3+
Mouse						
Normal ascitic fluid	2+	±	2+	±		
Cox B1 immune ascitic fluid	2+	±	3+	3+	3+	3+

[a] Symbols: ±, trace of fluorescence; 1–2+, low-level staining; 3–4+, brilliant staining of aggregates of viral antigen in the cytoplasm.

[b] Coxsackievirus.

tions of heterologous or normal sera (Fig. 2) or with uninfected cells (Fig. 3).

Homologous and heterologous FA titers of group B coxsackievirus antisera produced in three different species. Immune monkey and hamster sera for the group B coxsackieviruses and echovirus type 9 and group B coxsackievirus immune ascitic fluids prepared in mice were tested for homologous and heterologous immunofluorescent staining in infected BS-C-1 cells. As controls, normal sera from each species were examined for possible FA staining of infected and uninfected cells, and uninfected BS-C-1 cells were stained with immune and normal sera.

Indirect FA staining reactions of the monkey immune sera are shown in Table 2. Normal monkey sera failed to stain infected or uninfected cells. Antisera to coxsackievirus type B1 and echovirus type 9 showed only homologous staining. The other group B coxsackievirus immune sera showed low-level staining of cells infected with heterologous coxsackieviruses at serum dilutions of 1:8 to 1:64, but homotypic FA titers were appreciably higher. The homotypic staining revealed characteristic aggregates of viral antigen in the cytoplasm of the infected cells, whereas heterotypic staining reactions consisted of diffuse, low-level staining of the cytoplasm, and aggregates of viral antigen were not observed. Immune sera to coxsackievirus types B2 and B6 showed the greatest amount of heterotypic staining. Cells infected with echovirus type 9 showed no heterotypic staining with the group B coxsackievirus antisera.

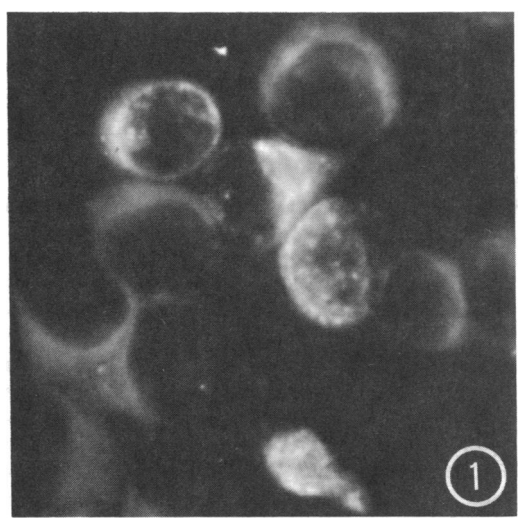

FIG. 1. *Indirect immunofluorescence staining reaction with group B coxsackievirus antiserum. Coxsackievirus type B4-infected BS-C-1 cells treated with coxsackievirus type B4 immune hamster serum and goat anti-hamster conjugate.*

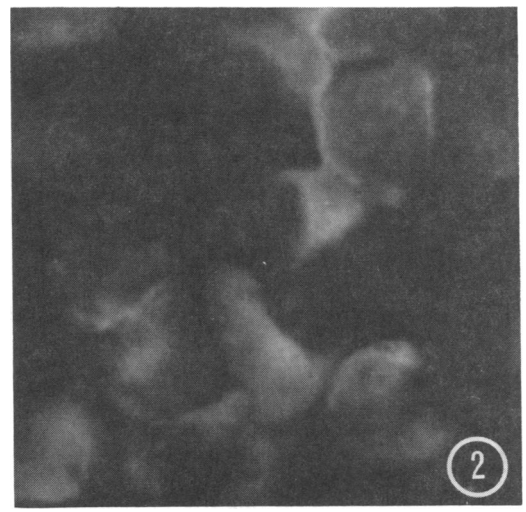

FIG. 2. *Indirect immunofluorescence staining reaction with group B coxsackievirus antiserum. Coxsackievirus type B4-infected BS-C-1 cells treated with coxsackievirus type B2 immune hamster serum and goat anti-hamster conjugate.*

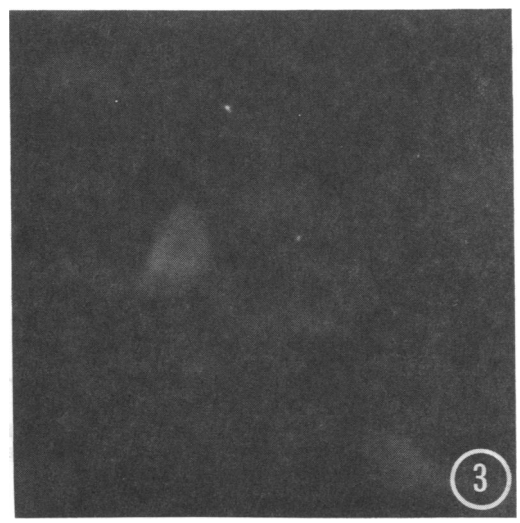

FIG. 3. *Indirect immunofluorescence staining reaction with normal serum. Uninfected BS-C-1 cells treated with normal hamster serum and goat anti-hamster conjugate.*

Table 3 shows the staining reactions of the immune hamster sera. The antisera produced in this species showed lower heterotypic staining titers than were seen with the monkey immune sera, but the homotypic titers also tended to be somewhat lower than those of the

TABLE 2. *Indirect immunofluorescence titration of monkey immune sera to group B coxsackieviruses employing BS-C-1 cells*

Cells infected with	Highest serum dilution showing immunofluorescence staining							
	Normal monkey serum	Monkey immune serum to coxsackieviruses						Monkey immune serum to echovirus 9
		B1	B2	B3	B4	B5	B6	
Uninfected BS-C-1 ..	<8[a]	<8	<8	<8	<8	<8	<8	<8
Coxsackievirus B1 ...	<8	**64/256**[b]	64[c]	32[c]	16[c]	8[c]	64[c]	<8
Coxsackievirus B2 ...	<8	<8	**256/1024**[b]	32[c]	16[c]	8[c]	64[c]	<8
Coxsackievirus B3 ...	<8	<8	64[c]	**128/1024**[b]	<8	<8	64[c]	<8
Coxsackievirus B4 ...	<8	<8	32[c]	8[c]	**32/128**[b]	16[c]	64[c]	<8
Coxsackievirus B5 ...	<8	<8	64[c]	32[c]	<8	**256/1024**[b]	64[c]	<8
Coxsackievirus B6 ...	<8	<8	64[c]	8[c]	8[c]	<8	**256/1024**[b]	<8
Echovirus 9	<8	<8	<8	<8	<8	<8	<8	**256/512**[b]

[a] Reciprocal of titer.

[b] Highest dilution showing 3 to 4+ staining of aggregates of iral antigen in the cytoplasm/highest dilution showing 1 to 2+ staining.

[c] Diffuse 1 to 2+ staining; no characteristic aggregates of viral antigen.

TABLE 3. *Indirect immunofluorescence titration of hamster immune sera to group B coxsackieviruses employing BS-C-1 cells*

Cells infected with	Highest serum dilution showing immunofluorescence staining							
	Normal hamster serum	Hamster immune serum to coxsackieviruses						Hamster immune serum to echovirus 9
		B1	B2	B3	B4	B5	B6	
Uninfected BS-C-1 ..	<8[a]	<8	<8	<8	<8	<8	<8	<8
Coxsackievirus B1 ...	8[b]	**32/128**[c]	16[b]	16[b]	16[b]	8[b]	8[b]	8[b]
Coxsackievirus B2 ...	<8	8[b]	**64/128**[c]	<8	<8	<8	<8	8[b]
Coxsackievirus B3 ...	<8	8[b]	8[b]	**32/128**[c]	8[b]	8[b]	8[b]	8[b]
Coxsackievirus B4 ...	<8	<8	8[b]	8[b]	**64/256**[c]	8[b]	8[b]	8[b]
Coxsackievirus B5 ...	<8	16[b]	16[b]	16[b]	16[b]	**128/256**[c]	16[b]	8[b]
Coxsackievirus B6 ...	<8	<8	<8	8[b]	<8	<8	**32/64**[c]	<8
Echovirus 9	8[b]	<8	8[b]	<8	<8	<8	<8	**256/512**[c]

[a] Reciprocal of titer.

[b] Diffuse 1 to 2+ staining; no characteristic aggregates of viral antigen.

[c] Highest dilution showing 3 to 4+ staining of aggregates of viral antigen in the cytoplasm/highest dilution showing 1 to 2+ staining.

monkey immune sera. Again, heterotypic staining consisted of diffuse, low-level staining of the cells, whereas homotypic staining revealed characteristic aggregates of viral antigen in the cytoplasm. Cross-reactivity with echovirus type 9 was absent or minimal.

Staining reactions of the immune mouse ascitic fluids for group B coxsackieviruses are shown in Table 4. The homotypic staining titers of the immune ascitic fluids approximated those of the hamster immune sera and the fluids showed low titers of heterotypic staining. The normal mouse ascitic fluid also showed low-level staining at the 1:8 and 1:16 dilutions, and normal and immune ascitic fluids stained uninfected BS-C-1 cells at dilutions of 1:8 and 1:16.

Identification of group B coxsackievirus isolates by indirect immunofluorescence staining. Field strains of coxsackieviruses, isolated and identified in this laboratory by standard procedures (12), were coded and handled as unknowns for attempted identification by FA staining. The isolates represented low-passage material in either rhesus MK cells or HFDK cells, and they were passaged into BS-C-1, MK, or HFDK cells for use in FA staining.

TABLE 4. *Indirect immunofluorescence titration of immune mouse ascitic fluid to group B coxsackieviruses employing BS-C-1 cells*

Cells infected with	Highest serum dilution showing immunofluorescence staining						
	Normal mouse ascitic fluid	Mouse immune ascitic fluid to coxsackieviruses					
		B1	B2	B3	B4	B5	B6
Uninfected BS-C-1	8[a]	8	8	16	8	8	16
Coxsackievirus B1	8	**32/128**[b]	8	16	16	8	8
Coxsackievirus B2	16	8	**256/256**[b]	16	8	8	8
Coxsackievirus B3	16	8	8	**128/256**[b]	8	8	8
Coxsackievirus B4	<8	<8	8	8	**32/64**[b]	8	8
Coxsackievirus B5	16	8	16	16	16	**128/256**[b]	8
Coxsackievirus B6	8	8	16	16	16	16	**64/256**[b]

[a] Reciprocal of titer; diffuse 1 to 2+ staining was seen in heterologous systems and with normal ascitic fluid.

[b] Highest dilution showing 3 to 4+ staining of aggregates of viral antigen in the cytoplasm/highest dilution showing 1 to 2+ staining.

The group B coxsackievirus antisera produced in hamsters were employed for indirect staining at working dilutions of 1:30, and reactivity of the hamster antibodies with viral antigen in the cells was detected through the use of fluorescein-conjugated anti-hamster globulins prepared from goat serum. Identification was based upon the demonstration of 3 or 4+ staining reactions.

Results of identification tests on isolates propagated in BS-C-1 cells are shown in Table 5. Approximately 93% of the 85 group B coxsackievirus isolates were identified correctly by FA staining at the first passage in BS-C-1 cells, and those not identifiable at the first passage were all correctly identified after a second passage in BS-C-1 cells. Some of the isolates had been stored for prolonged periods of time, and apparently infectivity titers were too low to permit identification at the first passage level. No incorrect identifications of group B coxsackievirus isolates were made, and none of the other viral immunotypes tested was incorrectly identified as group B coxsackieviruses. One field strain of coxsackievirus type B2 and one of type B4 failed to show a CPE in BS-C-1 cells; however both of these showed heavy staining with homologous antisera, and were correctly identified.

Limited attempts were also made to identify coxsackievirus isolates by FA staining using the cell culture systems routinely employed in this laboratory for isolation of enteroviruses, namely, MK and HFDK. Results are shown in Table 6. All of the group B coxsackievirus isolates tested were identified correctly by FA staining in both cell culture systems. In MK cells all of the isolates showed CPE, but less than one-half of them showed CPE in the HFDK cells; however, the isolates failing to show CPE were correctly identified by FA staining. It should be noted, however, that the infected HFDK cultures which did not show a CPE contained fewer cells showing specific staining than were seen in cultures with a

TABLE 5. *Identification of group B coxsackievirus isolates in BS-C-1 cells by indirect immunofluorescence staining*

Virus type	No. isolates tested	No. identified[a] (1st passage in BS-C-1)	Per cent identified
Coxsackievirus B1	16	12	75.0
Coxsackievirus B2	22	21	95.4
Coxsackievirus B3	9	8	88.8
Coxsackievirus B4	21	21	100.0
Coxsackievirus B5	16	16	100.0
Coxsackievirus B6	1	1	100.0
Total	85	79	92.9
Echovirus 9	1	0	0
Coxsackievirus A-9	2	0	0
Coxsackievirus A-13	1	0	0
Coxsackievirus A-16	3	0	0
Coxsackievirus A-18	1	0	0
Reovirus type 2	1	0	0
Total	9	0	0

[a] Number identified in tests against group B coxsackievirus hamster antisera. All isolates not identified in the first passage in BS-C-1 cells were identified on second passage.

TABLE 6. *Identification of group B coxsackievirus isolates in secondary monkey kidney (MK) and in human fetal diploid kidney (HFDK) cells by indirect immunofluorescence staining*

Virus type	Secondary MK		HFDK	
	No. of isolates identified[a]/no. tested	No. showing CPE[b]	No. of isolates identified[a]/no. tested	No. showing CPE[b]
Coxsackievirus B1	3/3	3	2/2	1
Coxsackievirus B2	4/4	4	4/4	0
Coxsackievirus B3	3/3	3	3/3	1
Coxsackievirus B4	3/3	3	2/2	1
Coxsackievirus B5	2/2	2	2/2	2
Coxsackievirus B6	2/2	2	2/2	1
Total	17/17	17	15/15	6
Coxsackievirus A9	0/1	1	0/1	1
Coxsackievirus A16	0/2	2	0/2	2
Total	0/3	3	0/3	3

[a] Number of isolates identified in tests against hamster antisera to group B coxsackieviruses.
[b] Cytopathic effect.

CPE. The group A coxsackievirus isolates tested in MK and HFDK cells did not show staining with antisera to the group B viruses.

DISCUSSION

These studies have confirmed the sensitivity and specificity of FA staining for identification of group B coxsackieviruses isolated in cell cultures. Identification is accomplished more rapidly by this method than by neutralization tests. Limited information derived from the present studies would suggest that FA staining reactions may be influenced less than are neutralization tests by the strain variations which may occur within immunotypes of the group B coxsackieviruses; some of the type B2 isolates which were readily identified by FA staining had shown "breakthrough" in neutralization tests, and identification could be accomplished only through the use of low dilutions of antisera. FA staining may prove to be as useful as complement fixation and immunodiffusion tests (15) for type-specific identification of antigenic variants of the coxsackieviruses.

Most workers who have utilized FA staining for identification of enteroviruses have employed the direct method with conjugated rabbit antisera (2–4, 6, 17). Our investigations have demonstrated that the indirect method, which requires only a single anti-species conjugate, is also feasible. Sommerville (18, 20) has used the indirect FA method with rabbit antisera for identification of group B coxsackieviruses in human leukocytes and cells from cerebrospinal fluids (18, 20). In our studies, rabbit antisera to the group B coxsackieviruses were found to be less satisfactory than monkey or hamster antisera or mouse immune ascitic fluids, due to their relatively low homologous FA staining titers.

Heterotypic FA staining was observed with antisera to the group B coxsackieviruses (but not with antisera to echovirus type 9), and the monkey antisera showed the highest heterotypic titers. With the hamster antisera and the immune mouse ascitic fluids, staining with heterotypic antisera was not appreciably greater than that seen with normal sera or uninfected cells. No consistent patterns of heterotypic staining were seen with antisera to the various coxsackievirus types. Some of the heterotypic staining may have been due to the presence in infected cells of group antigens, which have been demonstrated in group B coxsackievirus preparations through the use of immunodiffusion and complement fixation tests (13, 16). A good deal of the staining with heterotypic antisera may have been nonspecific; in this laboratory it has been generally observed that low dilutions (1:4 or 1:8) of conjugated or unconjugated antisera tend to give nonspecific staining in either direct or indirect FA tests. "Nonspecific" FA staining may also result from the use of antisera prepared by immunization with viral antigen derived from foreign host tissue or cell cultures containing foreign serum proteins. However, it is of interest that the immune mouse ascitic fluids, which were prepared by immunization with homologous, infected tissue, showed low-staining titers with heterologous antigens, and even with uninfected cell cultures.

The appearance of infected cells stained with heterotypic antisera differed from that of cells stained with homotypic antisera; heterotypic staining was a low-level (1 to 2+) diffuse staining of the cell, whereas homotypic staining revealed characteristic aggregates of viral antigen in the cytoplasm. Monkey and hamster antisera and mouse immune ascitic fluids could all be diluted to a point where they showed no heterotypic reactivity, but gave 3 to 4+ staining of cells infected with homologous virus, and showed characteristic aggregates of viral antigen. The demonstration of a 3 or 4+ staining reaction was considered essential for identification of viruses by FA staining.

The cross-testing of antisera and immune ascitic fluids for the group B coxsackieviruses

illustrated the importance of using immune reagents for which homotypic and heterotypic reactivity has been well defined in attempting to identify coxsackieviruses in cell cultures and tissues. The testing also revealed the pitfalls involved in using immune reagents at low dilutions in FA tests.

The studies reported herein were initiated primarily for evaluation and standardization of FA reagents to be employed in attempting to demonstrate group B coxsackieviruses or antigens in human tissues. Because little clinical material was available to the laboratory during the study period, only limited attempts were made to demonstrate antigen in human heart and brain. Tissues from two patients with isolations of coxsackievirus type B4 were studied. Hamster antisera and immune mouse ascitic fluids were used at the working dilutions which gave specific staining in cell culture systems, but FA staining of the human tissues with the type B4 immune reagents was no greater than that seen with heterologous reagents, despite the fact that coxsackievirus type B4 had been isolated from the tissues. Thus, recognition and identification of coxsackieviruses in tissues may prove to be more difficult than identification in cell culture materials. Additional evaluation of immune reagents must be performed using coxsackievirus-infected tissues before valid conclusions can be drawn regarding the FA staining of human tissues with antisera to group B coxsackieviruses, and FA staining reactions of infected tissues should be better correlated with viral isolation attempts or with attempts to demonstrate coxsackievirus antigen by other immunological procedures.

ACKNOWLEDGMENTS

We are indebted to James D. Woodie for assistance and consultation provided during the course of these studies.

This study was supported by Public Health Service grants GM-01038-07 from the National Institute of General Medical Sciences and AI-01475 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Bates, H. R. 1970. Coxsackievirus B3 calcific pancarditis and hydrops fetalis. Amer. J. Obstet. Gynecol. **106:** 629-630.
2. Burch, G. E., K. C. Chu, H. L. Colcolough, and R. S. Sohal. 1969. Immunofluorescent localization of coxsackievirus B antigen in the kidney observed at routine autopsy. Amer. J. Med. **47:**36-42.
3. Burch, G. E., and H. L. Colcolough. 1969. Progressive coxsackie viral pancarditis and nephritis. Ann. Intern. Med. **71:**963-970.
4. Burch, G. E., S. C. Sun, H. L. Colcolough, R. S. Sohal, and N. P. De Pasquale. 1967. Coxsackie B viral myocarditis and valvulitis identified in routine autopsy specimens by immunofluorescent techniques. Amer. Heart J. **74:**13-23.
5. Chaudhary, R. K., and J. C. N. Westwood. 1970. Detection of crossreactions by immunofluorescence within the picornavirus group. Can. J. Microbiol. **16:**601-604.
6. Hatch, M. H. 1963. Identification of coxsackie and ECHO virus isolates with fluorescent antibody. Proc. Soc. Exp. Biol. Med, **114:**161-165.
7. Lennette, E. H., J. D. Woodie, K. Nakamura, and R. L. Magoffin. 1965. The diagnosis of rabies by the fluorescent antibody method (FRA) employing immune hamster serum. Health Lab. Sci. **2:**24-34.
8. Lim, K. A., and M. Benyesh-Melnick. 1960. Typing of viruses by combinations of antiserum pools. Application to typing of enteroviruses (coxsackie and ECHO). J. Immunol. **84:**309-317.
9. Melnick, J. L., and B. Hampil. 1970. WHO collaborative studies on enterovirus reference antisera, third report. Bull. World Health. Organ. **42:**847-863.
10. Melnick, J. L., and H. A. Wenner. 1969. Enteroviruses, p. 529-602. *In* E. H. Lennette, and N. J. Schmidt, (ed.), Diagnostic procedures for viral and rickettsial diseases, 4th ed. American Public Health Association Inc., New York.
11. Riggs, J. L., and G. C. Brown. 1962. Application of direct and indirect immunofluorescence for identification of enteroviruses and titrating their antibody. Proc. Soc. Exp. Biol. Med. **110:**833-837.
12. Schmidt, N. J. 1969. Tissue culture technics for diagnostic virology, p. 79-178. *In* E. H. Lennette and N. J. Schmidt, (ed.), Diagnostic procedures for viral and rickettsial diseases, 4th ed. American Public Health Association, New York.
13. Schmidt, N. J., J. Dennis, L. H. Frommhagen, and E. H. Lennette. 1963. Serologic reactivity of certain antigens obtained by fractionation of Coxsackie viruses in cesium chloride density gradients. J. Immunol. **90:**654-662.
14. Schmidt, N. J., R. W. Guenther, and E. H. Lennette. 1961. Typing of ECHO virus isolates by immune serum pools. The "Intersecting Serum Scheme." J. Immunol. **87:**623-626.
15. Schmidt, N. J., and E. H. Lennette. 1970. Antigenic variants of coxsackievirus type A24. Amer. J. Epidemiol. **91:**99-109.
16. Schmidt, N. J., E. H. Lennette, and J. Dennis. 1965. Gel double diffusion studies with group B and group A, type 9, coxsackieviruses. III. Antigen-antibody absorption tests. J. Immunol. **94:**482-491.
17. Shaw, E. D., A. Newton, A. W. Powell, and C. J. Friday. 1961. Fluorescent antigen-antibody reactions in coxsackie and ECHO enteroviruses. Virology **15:**208-210.
18. Sommerville, R. G. 1966. Rapid identification of neurotropic viruses by an immunofluorescent technique applied to cerebrospinal fluid cellular deposits. Arch. Gesamte Virusforsch. **19:**63-69.
19. Sommerville, R. G. 1967. The production of fluorescent antibody reagents for virus diagnosis in the albino mouse. I. Hyperimmune anti-species serum. Arch. Gesamte Virusforsch. **20:**445-451.
20. Sommerville, R. G., and P. S. Macfarlane. 1964. The rapid diagnosis of virus infections by immunofluorescent techniques applied to blood leucocytes. Lancet **i:**911-912.
21. Zalan, E., A. E. Kelen, and N. A. Labzoffsky. 1965. Immunofluorescence studies on Coxsackie group A viruses. I. Localization of viral antigen in infected primary human amnion cells. Arch. Gesamte Virusforsch. **15:**668-680.